Gas-Liquid Chromatography of Lipids, Carbohydrates and Amino Acids[1]

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Abstract

Gas-liquid chromatography is primarily a powerful separating tool. Compounds can be trapped as they emerge from the GLC apparatus for analysis by mass spectrometry and infrared or ultraviolet spectrophotometry, or the emerging separated components may flow directly into one of these instruments making positive identification of the components possible. Quantitative analyses of fats and oils are possible when certain requirements are observed. A combination of fatty acid, triglyceride and sterol analyses offers promise of a rapid means for the identification of fats and oils and their admixture or adulteration. Progress has been made on the preparation of derivatives of carbohydrates and amino acids such that these compounds may soon be analyzed as readily by GLC as lipids are today.

Introduction

GAS-LIQUID CHROMATOGRAPHY is primarily a means for separating mixtures and cannot of itself identify a compound but offers only a tentative identification based on similar retention with a known compound. On the other hand, spectroscopic or x-ray techniques are capable of identifying a compound but usually require highly purified samples. Therefore, it is not surprising that each technique should become dependent on the other; gas chromatography to separate a pure compound from a mixture or to show that a compound is indeed a single entity; spectroscopy or x-ray techniques for positive identification of the separated or pure compound.

Discussion

Infrared and mass spectrometers have been connected in tandem with gas chromatographs (2,4,13,14,18,23,24,27,28). As compounds are separated from mixtures and eluted from the gas chromatograph, they pass directly into these instruments and are identified. In the analysis of fatty acids by this technique, infrared spectroscopy has left something to be desired for two reasons: the more common fatty acids exhibit only small differences in their spectra, and the resolution of the required rapid scan instruments has generally not been sufficient to show these small differences. The mass spectrometer in tandem has been a little more useful for the identification of the fatty acids. However, getting one of these high boiling compounds into the instrument and then flushed out again before the next component enters has given considerable trouble to most investigators. As a result, tandem techniques are not in common usage for lipid analysis. A more common procedure is to trap the component as it leaves the gas chromatograph and then analyze the trapped component by infrared or mass spectroscopy.

One of the more successful separations and identification employing a gas chromatograph and mass spectrometer in tandem was published last year by Ryhage (19). Butterfat methyl esters were separated on a capillary column. The scanning time of each peak was about 3 sec as it was eluted. Sixty-six peaks were recorded on the chromatogram and of these, 52 were identified from their mass spectra. The results were obtained in one day.

One of the first applications of GLC in lipid analysis was its use to separate the fatty acids. Later, it was found advantageous to convert the acids to their methyl esters. The GLC analysis of fatty acids as their methyl esters has developed until today it has acceptance as the best method for determination of composition. There are still problems of separation with very complex mixtures such as milk fat, fish oil and fat from various animal organs but for the more common fats of trade, the analysis can be quite precise and accurate.

Data obtained from the AOCS Smalley Gas Chromatography collaborators shows that instrument, type of stationary liquid or support used in the column, length or diameter of the column, type of detector or a wide range of operating conditions will not result in significant differences in the analysis of a fat or oil provided one uses a reference sample for standardization. Earlier analyses by this group indicated that the standard deviation was greater for those employing the flame ionization detector when compared to those employing thermal conductivity. However, these differences have since disappeared. It was found that there are two principal reasons for the lack of precision between laboratories. First, improper operation of the gas chromatograph or the recorder was shown by inspection of gas chromatograms from each AOCS Smalley collaborator and members of the Instrumental Techniques Gas Chromatography Subcommittee. For example, one operator employed conditions in which the methyl esters of an oil were eluted so quickly that methyl stearate was eluted in 1½ min. The chart speed was such that very narrow base peaks were drawn. If a good integrator had been used, the results probably would have been satisfactory but the operator reported that the area was determined by triangulation. Another chromatogram indicated a shifting base line upon attenuation because the original baseline was not at the recorder zero. The chromatogram exhibited stepping which indicated improper setting of the gain and damping adjustment of the recorder or possibly a faulty amplifier. These are just two examples where certain practices are employed which do not lend themselves to reproducible quantitative results. Poor results could be correlated with chromatograms which showed a disregard for good operating practice rather than the use of a great variety of operating conditions where reasonable care was taken. The second reason for lack of precision lies in not employing reference mixtures to either check the response of the detector or to obtain response factors to correct the data. Reference mixtures are available commercially which approximate the composition of many oils and when these are

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amount of 56, these peaks are absent in olive oil. The chromatograms of the sterols of these two oils show only two major peaks for olive oil but peanut oil exhibits three major peaks. Again if these prove to be typical profiles of peanut and olive oil from a number of sources and from different varieties, either the triglyceride or the sterol analysis would offer distinguishing features. The combination of fatty acid, triglyceride and sterol composition seems to offer great promise in identification of oils and possibly their admixtures or adulteration.

One may obtain all three analyses in little more time than that required for the fatty acid composition. To obtain the data with a single gas chromatograph, it would be necessary to change columns but with two instruments or one instrument equipped with two separately controlled ovens, the data may be obtained rapidly. For the triglyceride determination, we employed a 3 ft × ⅛-in. stainless steel tube packed with 3% JXR on 100–120 mesh Gas Chrom Q. The initial temperature was 210 C and the oven temperature programmed at 2 C/min to a temperature of 310 C. Helium carrier flow was 65 cc/min. The sterol separation was obtained on the same column but the oven was operated isothermally at a temperature of 200 C. The methyl esters of the fatty acids were analyzed on an 8 ft × ⅛-in. stainless steel tube packed with 10% diethylene glycol succinate on 60–70 mesh Anakrom A. The oven temperature was held isothermally at 175 C and the helium carrier flow was 65 cc/min. A hydrogen flame detector was employed in both instruments.

The triglyceride analysis takes about 1 hr. While the sample was being chromatographed, a transesterification of the triglyceride to the methyl esters was performed by the procedure of Luddy et al. (15) in 5 to 10 min. The reaction mixture contained the unsaponifiables as well as the methyl esters. The reaction mixture was streaked on a thin-layer plate and developed for about 20 min. The two regions containing the methyl esters and the sterols were scraped off the plate and extracted separately. By this time the triglyceride analysis was completed and the sterols were injected onto the same column used for the triglyceride analysis while the methyl esters were injected on the polyester column. About 30 min are required for these analyses, which means that a triglyceride, sterol and fatty acid analysis were completed in about 1½ hr.

Carbohydrates

The application of gas chromatography to the separation of carbohydrates and related polyhydroxy compounds has tended to lag behind the development of this technique with most other classes of compounds. A major difficulty has been the preparation of volatile derivatives of the polyhydroxy compounds by rapid and general methods. In 1963, Sweeley et al. (22) made a significant contribution when they reported the separation of the trimethylsilyl ether (TMS) derivative of almost 100 carbohydrates and related compounds. The TMS derivatives are formed usually in about 5 min at room temperature. The products obtained from pure single anomers of pentoses and hexoses generally show a single peak on GLC. Almost all other previously reported derivatives resulted in multiple peaks during GLC.

Because of its speed and resolving power, GLC is rapidly replacing other techniques in the fermentation industry as it has in so many other industries. Most notable is in the analyses of the volatile constituents. However, the development of the procedure for preparing TMS derivatives has stimulated interest in GLC for the analysis of carbohydrates. Sweeley removed water from aqueous sugar solutions before silylation. Brobst and Lott, Jr. (3) modified the procedure such that a limited amount of water was permitted. This procedure was applied to the analysis of corn syrup carbohydrates without extensive removal of water. Marinelli and Whitney (16) used this method to determine the carbohydrates of not only corn syrup but beer and wort carbohydrates as well.

Most carbohydrates show multiple peaks due to mutarotation of the sugars in aqueous equilibrium mixtures. This somewhat complicates the chromatograms but at times they are helpful in identifying unknown carbohydrates since reference could be made to more than one retention. A monosaccharide may form as many as four glycosides as a result of anomeric and ring isomerization. This can be avoided by reduction to their alditols with sodium borohydride (20). The alditols are then acetylated with acetic anhydride. The alditols cannot anomerize and the separation of the alditol derivative eliminates the problem of multiple peaks.

The possible application of the procedure would include the analysis of sugar mixtures, the identification of hydrolysis products of barley, wort and beer polysaccharides to elucidate their structure, studies of the assimilation of carbohydrates by microorganisms, and investigations of the nature and action of carbohydrases and many others. It might be mentioned here that Supina et al. (21) have recently suggested the use of dimethylsilyl (DMS) derivatives of the carbohydrates. They report equally good separations but in a shorter retention time and at a lower column temperature.

Amino Acids

A review by Weinstein (26) describes the efforts to develop quantitative gas chromatographic methodology for the analysis of amino acids as volatile derivatives. Recently Gehrke and Stallings (7) published a method for the quantitative determination of 20 natural protein amino acids and applied the procedure to the analysis of biological materials. In the latter instance, the requirements of sample preparation for GLC are greater than for the conventional ion-exchange analysis. Lipids, amines and sugars which would give rise to interfering peaks in the chromatogram during gas chromatography must be removed and the sample converted to the N-trifluoroacetyl n-butyl esters. The time for the removal of these interfering substances will depend on the compounds present but the conversion and subsequent analysis by GLC takes about 4 or 5 hrs. Therefore, an analysis is not much, if at all, faster than the conventional ion-exchange procedure and certainly not as fast if one can use Ligand Exchange Chromatography (8) where a similar amino acid mixture was analyzed in 2 hr and the additional sample treatment for gas chromatography was not necessary.

The development by Gehrke and Stallings is an excellent piece of work and will undoubtedly stimulate more interest in GLC of amino acids but it is not the simple, rapid analysis that may be accomplished in an hour as has been reported in some instrument company literature. Rather, this proce-

dure as stated by Gehrke is a "logical step in the evolution of a GLC method for quantitative determination of amino acids." Gas chromatography offers the advantage of rapid separation of difficult multicomponent mixtures, use of small samples, accuracy and simplicity. For certain amino acid analyses, GLC is today the equal of the older ion exchange procedure but it has one distinct advantage, namely, the analyses can be attained with a relatively cheaper instrument that may also be used for the analyses of many other materials.

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